

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
14 February 2002 (14.02.2002)

PCT

(10) International Publication Number  
**WO 02/12201 A1**

(51) International Patent Classification<sup>7</sup>: **C07D 241/08**

(21) International Application Number: PCT/US01/24281

(22) International Filing Date: 2 August 2001 (02.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/223,075 4 August 2000 (04.08.2000) US

(71) Applicant: **DMI BIOSCIENCES, INC.** [US/US]; 3601  
S. Clarkson Street #420, Englewood, CO 80110-3948 (US).

(72) Inventors: **RAO, Nagaraja, K., R.**; 17 Dryburgh Avenue,  
Birchgrove, Cardiff CF4 4QN (GB). **TURNER, Stephen**;  
24, Ashbourne, Great Hollands, Bracknell, Berkshire  
RG12 8SG (GB).

(74) Agent: **CROOK, Wannell, M.**; Sheridan Ross P.C., Suite  
1200, 1560 Broadway, Denver, CO 80202-5141 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

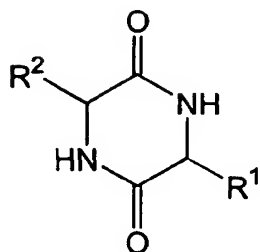
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD OF SYNTHESIZING DIKETOPIPERAZINES



(57) Abstract: The invention provides a method of synthesizing a diketopiperazine of the formula: wherein R<sup>1</sup> is CH<sub>2</sub>COR<sup>3</sup>, or -CH<sub>2</sub>CH<sub>2</sub>COR<sup>3</sup>, R<sub>2</sub> is the side chain of an amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine; R<sup>3</sup> is -OH, -NH<sub>2</sub>, -OR<sup>4</sup>, or -NR<sup>4</sup>R<sup>4</sup>; and each R<sup>4</sup> is independently an alkyl, aryl, alkylaryl, or arylalkyl.

WO 02/12201 A1

## METHOD OF SYNTHESIZING DIKETOPIPERAZINES

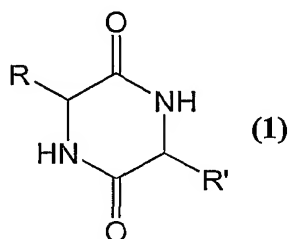
### FIELD OF THE INVENTION

This invention relates to a method of synthesizing diketopiperazines.

5

### BACKGROUND

Many diketopiperazines of the general formula (1) are known to possess

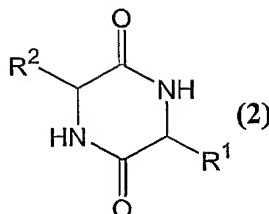


10 pharmacological and other biological activities, and libraries of such compounds have been synthesized to identify other diketopiperazines having beneficial biological and medicinal activities. See, *e.g.*, Smith et al., *Bioorg. Med. Chem Letters*, 8:2369-2374 (1998); Nitecki et al., *J. Org. Chem.*, 33:864-866 (1968); U.S. Patent No. 5,817,751; and PCT application WO 96/00391. Diketopiperazines of formula (1) have been synthesized by a variety of  
15 methods, but, a thermal cyclization of the corresponding dipeptide to produce the cyclic product seems to be the method of choice because of its simplicity. In addition, the method is known to produce sterically-pure product (Nitecki et al., *J. Org. Chem.*, 33:864-866 (1968)).

However, several groups of workers have encountered difficulty in employing the  
20 thermal cyclization method to synthesize diketopiperazines containing an aspartic acid residue. The inability of the dipeptide to form a cyclic product has been attributed to the presence of the free  $\beta$  carboxyl group of the aspartic acid. Similar difficulties would be expected synthesizing diketopiperazines containing a glutamic acid. Consequently, a new strategy to synthesize diketopiperazines wherein one of the amino acid is aspartic acid or  
25 glutamic acid is needed.

### SUMMARY OF THE INVENTION

The present invention provides the needed method. In particular, the invention provides a method of synthesizing diketopiperazines of the following formula:



wherein:

$R^1$  is  $-\text{CH}_2\text{COR}^3$ , or  $-\text{CH}_2\text{CH}_2\text{COR}^3$ ;

5  $R^2$  is the side chain of an amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine;

$R^3$  is  $-\text{OH}$ ,  $-\text{NH}_2$ ,  $-\text{OR}^4$ ,  $-\text{NHR}^4$ , or  $-\text{NR}^4\text{R}^4$ ; and

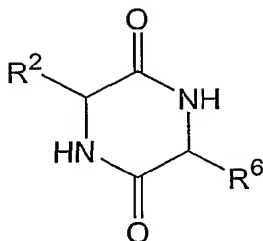
10 each  $R^4$  is independently an alkyl, aryl, alkylaryl, or arylalkyl.

The method comprises providing a first amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine. The first amino acid  
15 is optionally protected with one or more protecting groups so as to prevent unwanted side reactions.

The first amino acid is reacted with a second amino acid derivative which is either an aspartic acid derivative of the following formula  $\text{NH}_2\text{CH}(\text{CH}_2\text{COOR}^5)\text{COOH}$ , or a glutamic acid derivative of the following formula  $\text{NH}_2\text{CH}(\text{CH}_2\text{CH}_2\text{COOR}^5)\text{COOH}$ , wherein  
20  $R^5$  is a lower alkyl or alkylaryl. The amino group or  $\alpha$  carboxyl group of the second amino acid derivative is optionally protected with a protecting group so as to prevent unwanted side reactions. The reaction of the first amino acid and the second amino acid derivative takes place under conditions effective to form a dipeptide.

The resulting dipeptide is then cyclized to form a diketopiperazine having the  
25 formula:

3



5

wherein:

$R^6$  is  $-\text{CH}_2\text{COOR}^5$ , or  $-\text{CH}_2\text{CH}_2\text{COOR}^5$ .

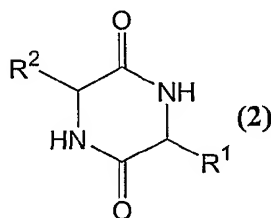
Then, the  $R^5$  group is removed from the diketopiperazine to give a compound of formula 2 wherein  $R^3$  is  $-\text{OH}$ . Diketopiperazines of formula 2 wherein  $R^3$  is other than  $-\text{OH}$  (i.e., amides and esters) may be made from this diketopiperazine.

10

#### DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides a method of synthesizing diketopiperazines of the following formula:

15



wherein:

$R^1$  is  $-\text{CH}_2\text{COR}^3$ , or  $-\text{CH}_2\text{CH}_2\text{COR}^3$ ;

$R^2$  is the side chain of an amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine;

$R^3$  is  $-\text{OH}$ ,  $-\text{NH}_2$ ,  $-\text{OR}^4$ ,  $-\text{NHR}^4$ , or  $-\text{NR}^4\text{R}^4$ ; and  
each  $R^4$  is independently an alkyl, aryl, alkylaryl, or arylalkyl.

25

By "side chain" of an amino acid is meant that portion of the amino acid attached to

the common  $\text{NH}_2\text{-CH-COOH}$  backbone of all of the amino acids listed above. For instance, the side chain of glycine is  $\text{-H}$ , the side chain of alanine is  $\text{-CH}_3$ , and the side chain of serine is  $\text{-CH}_2\text{OH}$ .

By "alkyl" is meant a straight-chain or branched-chain alkyl containing 1-30 carbon atoms, preferably 1-18 carbon atoms. "Lower alkyl" means a straight-chain or branched chain alkyl containing 1-6 carbon atoms.

By "aryl" is meant an aromatic group having at least one aromatic ring (e.g., phenyl).

By "alkylaryl" is meant a lower alkyl having attached thereto an aryl (e.g.,  $\text{-CH}_2\text{C}_6\text{H}_5$  or  $\text{-CH}_2\text{CH}(\text{C}_6\text{H}_5)\text{CH}_3$ ).

By "arylalkyl" is meant an aryl having attached thereto a lower alkyl (e.g.,  $\text{-C}_6\text{H}_4\text{-CH}_3$ ).

The synthesis of the diketopiperazines utilizes standard solution-phase or solid-phase peptide synthetic methods which are well known in the art. Preferably, the synthesis of the diketopiperazines utilizes solid-phase peptide synthetic methods.

The first step of the method of the invention comprises providing a first amino acid. The first amino acid is selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine. These amino acids, which may be in their D- or L-enantiomeric form, are commercially available or can be made by methods well known in the art (see, e.g., Williams, *Synthesis Of Optically Active  $\alpha$ -Amino Acids* (Pergammon Press, 1989)).

The first amino acid is also preferably protected with one or more protecting groups to prevent unwanted side reactions during the synthesis. Such protecting groups, and methods for attaching and removing them, are well known in the art. See, e.g., Green and Wuts, *Protective Groups In Organic Chemistry* (Wiley 1992) and Grant, *Synthetic Peptides: A User's Guide* (Freemen 1992).

The first amino acid is reacted with an aspartic acid derivative of the following formula  $\text{NH}_2\text{CH}(\text{CH}_2\text{COOR}^5)\text{COOH}$  or a glutamic acid derivative of the following formula  $\text{NH}_2\text{CH}(\text{CH}_2\text{CH}_2\text{COOR}^5)\text{COOH}$ , wherein  $\text{R}^5$  is a lower alkyl or alkylaryl. Preferably  $\text{R}^5$  is benzyl ( $\text{-CH}_2\text{C}_6\text{H}_5$ ; Bz). The benzyl group has been found not only to protect the side-chain

carboxyls of these amino acids, but also to facilitate cyclization of the dipeptide. Furthermore, the benzyl can be removed from the dipeptide under neutral conditions which prevents racemization of the chiral center (carbons bearing the R<sup>1</sup> and R<sup>2</sup> groups).

The aspartic and glutamic acid derivatives NH<sub>2</sub>CH(CH<sub>2</sub>COOR<sup>5</sup>)COOH and  
5 NH<sub>2</sub>CH(CH<sub>2</sub>CH<sub>2</sub>COOR<sup>5</sup>)COOH are commercially available or may be prepared by known methods (see, e.g., Bodansky and Bodansky, *The Practice of Peptide Synthesis*, pages 63-66 (2nd ed., Springer-Verlag, 1994). The amino group or the α carboxyl of the aspartic and glutamic acid derivatives can optionally be blocked with a standard protecting group (see above) in order to prevent unwanted side reactions.

10 As noted above, the synthesis of the diketopiperazines preferably utilizes solid-phase peptide synthetic methods. The first amino acid or the aspartic or glutamic acid derivative is attached to a solid support through its α carboxyl for solid-phase synthesis. The solid support may be any solid support which is compatible with peptide synthesis, such as those described in Grant and Atherton, *Solid Phase Peptide Synthesis: A Practical Approach* (IRL  
15 Press 1989). Suitable solid supports are available commercially or can be prepared by standard methods. See PCT application WO 96/00391. The solid support may contain linker or spacer molecules which anchor the first amino acid or the aspartic acid or glutamic acid derivative to the support surface. A variety of linkers with different properties are well known in the art. See, e.g., Grant, *Synthetic Peptides: A User's Guide* (Freeman 1992) and  
20 PCT application WO 96/00391. The linker will typically include a functional group to which the first amino acid or the aspartic acid or glutamic acid derivative is attached.

Preferably, the first amino acid is attached to the solid support and, prior to coupling the aspartic acid or glutamic acid derivative to the first amino acid, the protecting group, if present, on the α amino group of the bound first amino acid is removed. The removal of the  
25 protecting group of any side-chain amino groups should be avoided, however, so conditions must be chosen to deprotect the α amino group without deprotecting any side chain amino groups. Suitable deprotection conditions are known in the art. For example, removal of 9-fluorenylmethyloxycarbonyl may be performed with 20% to 55% of a secondary amine base, such as piperidine, in a polar, aprotic solvent, such as dimethylformamide, methylene  
30 chloride or N-methylpyrrolidine. Diisopropyl silane is preferably added to prevent transesterification during deprotection, which can be pronounced in large scale preparations.

The reaction between the first amino acid and the aspartic or glutamic acid derivative takes place under conditions effective to produce a peptide bond so that a dipeptide is formed. These conditions are well known in the art. For instance, a coupling catalyst (such as 2-(1H-benzotriazole-1-yl)-1,2,3,3-tetramethyluroniumtetrafluoroborate, benzotriazole-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexaphosphate, 1-hydroxybenzotriazole, diisopropylamine, dicyclohexylcarbodiimide, ) may be used to effect formation of the dipeptide. Typically, an excess of the coupling catalyst is used, with quantities ranging from 2 to 10 equivalents or more. Often the degree of excess is determined with respect to the reactivity of the chemical species being coupled. Polar, aprotic solvents (such as dimethylformamide, N-methylpyrrolidine, methylene chloride and dimethylsulfoxide) are preferred. Reaction times may vary from one-half hour to overnight, and temperatures may vary from room temperature to reflux.

Next, if the dipeptide is bound to a solid support, it is removed from the solid support using standard procedures well known in the art. The conditions effective to remove the dipeptide from the solid support will be depend on the solid support and linker chosen. Generally, the peptide will be removed by acid hydrolysis using a strong acid, such as trifluoroacetic acid.

The dipeptide is then cyclized to form a diketopiperazine; this diketopiperazine will have the side-chain carboxyl of the aspartic acid or glutamic acid derivative still in the ester form. Cyclization is accomplished by heating the dipeptide under neutral conditions. Typically, the dipeptide will be heated at from about 80°C to about 180°C, preferably at about 120°C. The solvent will be a neutral solvent. For instance, the solvent may comprise an alcohol (such as butanol, methanol, ethanol, and higher alcohols, but not phenol) and an azeotropic co-solvent (such as toluene, benzene, or xylene). Preferably, the alcohol is butan-2-ol, and the azeotropic co-solvent is toluene. The heating is continued until the reaction is complete, and such times can be determined empirically. Typically, the dipeptide will be cyclized by refluxing it for about 8-24 hours, preferably about 18 hours.

Finally, the R<sup>5</sup> group is removed from the diketopiperazine by methods well known in the art for removing protecting groups (see above). Preferably, when the R<sup>5</sup> group is benzyl, it is removed from the diketopiperazine by hydrogenation using a palladium on

carbon catalyst (Pd/C). The use of strong acids (mineral acids, such as sulfuric or hydrochloric acids), strong bases (alkaline bases, such as potassium hydroxide or sodium hydroxide), and strong reducing agents (*e.g.*, lithium aluminum hydride) should be avoided in order to maintain the chirality of the final compound.

5        Attempts to prepare Asp-Ala diketopiperazine (**5**) using prior art methods failed to give the desired product. In particular, literature procedures describing the synthesis of (**5**) from formate salts of dipeptides failed to give any product when the side chain contained a free carboxylic acid. By contrast, use of the method of the present invention gives the desired diketopiperazines. When R<sup>5</sup> is benzyl, yields of about 90% with a purity greater than 95%  
10        are obtained. Further, the benzyl esters of the dipeptides are easily isolated from the reaction medium and are easily purified by crystallization which, in turn, lends itself to obtaining an optically pure product.

      Once the R<sup>5</sup> group has been removed, the free acid can be derivatized, if desired, to form standard derivatives, such as amides and esters. Methods which can be used to convert  
15        the free acid to an amide or ester are well known in the art.

      The diketopiperazines of formula (**2**) have been found to inhibit the effects of platelet activating factor (PAF). See co-pending provisional application 60/222,849, filed August 4, 2000, the complete disclosure of which is incorporated herein by reference. For instance, a disease or condition mediated by PAF can be treated, platelet aggregation can be inhibited,  
20        the production and/or release of interleukin 8 (IL-8) by cells can be inhibited, and a disease or condition mediated by IL-8 can be treated with diketopiperazines of formula (**2**). Diseases or conditions mediated by PAF include acute respiratory distress syndrome, allergies, arthritis, asthma, autoimmune diseases, bronchitis, cardiovascular disease, Crohn's disease, cystic fibrosis, emphysema, gastrointestinal ulceration, inflammation, inflammatory bowel  
25        disease, ischemia, multiple organ dysfunction syndrome, myocardial infarction, neoplastic diseases, ophthalmic inflammation, pain, psoriasis, respiratory infections, sepsis, shock and ulcerative colitis, and any of these diseases or conditions can be treated with diketopiperazines of formula (**2**). Diseases or conditions mediated by IL-8 include acute  
30        respiratory distress syndrome, allergies, arthritis, asthma, autoimmune diseases, bronchitis, cancer, Crohn's disease, cystic fibrosis, emphysema, endocarditis, gastritis, inflammatory bowel disease, ischemia reperfusion, multiple organ dysfunction syndrome, neoplastic



diseases, nephritis, pancreatitis, respiratory viral infections, sepsis, shock, ulcerative colitis, and other inflammatory disorders, and any of these diseases or conditions can also be treated with diketopiperazines of formula (2).

5

## EXAMPLES

### Example 1: Synthesis of Asp-Ala Diketopiperazine (5)

Wang resin having 9-fluorenylmethyloxycarbonyl-protected alanine (Ala-Fmoc) attached thereto (3 grams (g), 2.52 mmol, 1 equivalent, NovaBiochem, La Jolla, CA) was transferred to a clean round-bottom, 100 mL flask, and a solution of piperidine (12 mL) in dimethylformamide (DMF; 18 mL) was added to the resin in the flask. The solution was swirled for 1 hour, and the resin was isolated in a sintered glass funnel. The resin was washed with DMF (3 x 30 mL) followed by dichloromethane (DCM; 3 x 30 mL) and allowed to dry under vacuum for 5 minutes. Preferred protecting groups are 9-fluorenylmethyloxycarbonyl (Fmoc) and t-butoxycarbonyl (Boc).

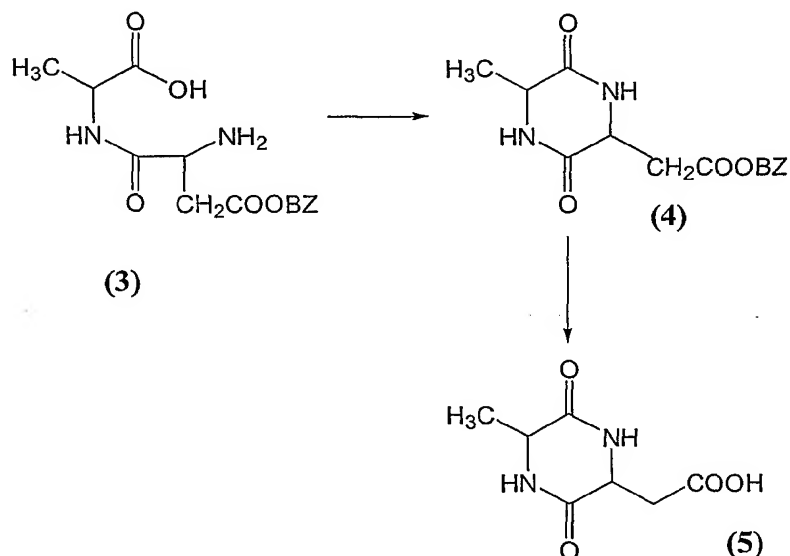
The partially-dried resin was transferred into a clean round-bottom, 100 mL flask, and DMF (10 mL) was added. Then, Boc-Asp(OBz)OH (3.25 g, 10.07 mmol, 4 equivalents; Aldrich) was added, followed by diisopropylamine (2.83 mL, 2.04 g, 20.19 mmol, 8 equivalents) and 2-(1H-benzotriazole-1-yl)-1,2,3,3-tetramethyluroniumtetrafluoroborate (TBTU; 3.24 g, 10.09 mmol, 4 equivalents, Acros, Loughborough Leicestershire, UK). The slurry was allowed to react under anaerobic conditions over 12 hours. At the end of this time, the resin showed a negative ninhydrin test, indicating the completion of the coupling reaction. The resin was vacuum filtered and washed with DMF (3 x 30 mL) followed by DCM (3 x 30 mL). The resin was allowed to dry at room temperature under vacuum for 10 minutes before transferring it into a clean round-bottom, 100 mL flask.

Trifluoroacetic acid (TFA; 16.5 mL) was added to the dried resin and, upon its addition, the resin turned a red color. After swirling the resin for a further 30 minutes, TFA was removed by filtration, and the resin was washed with DCM (4 x 20 mL). The organic components were pooled, and toluene (20 mL) was added. The combined organic materials were evaporated to dryness under vacuum. Traces of TFA were removed by the addition of toluene and evaporation. The process was repeated until all TFA had been removed. This procedure resulted in a product as a pale yellow oil whose NMR and mass

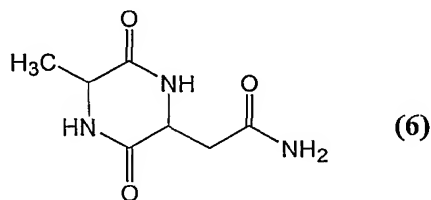
spectrophotometric data were consistent with the expected dipeptide benzyl ester whose structure (3) is shown below.

The dipeptide 3 was dissolved in butan-2-ol (40 mL) and diluted with toluene (60 mL). This solution was allowed to reflux for 24 hours. At the end of this period, the solution was allowed to cool to room temperature. It was then concentrated on a rotary evaporator, while maintaining the temperature at 50°C. Upon concentration, a white solid precipitated, and the precipitate was removed by filtration. The precipitate was washed with toluene (10 mL) and dried. The residue (0.650 g) gave a negative ninhydrin test. It was, then, crystallized from hot methanol. The spectroscopic and analytical results for the crystallized product confirmed its structure to be the desired compound - Asp-Ala diketopiperazine-benzyl ester shown below (4).

This compound (400 mg) was dissolved in methanol (250 mL), and palladium on carbon catalyst (Pd/C; 10%, 0.4g; Fluka Chemicals, Dorset UK) was added carefully. The flask was purged with hydrogen and kept at a positive hydrogen pressure. The solution was kept in this atmosphere for at least 4 hours. The catalyst was removed with a filtering aid (celite) and washed with methanol. The methanol washings were combined, and the solvent was removed (yield 200 mg). Mass spectrometer and NMR analysis showed that the free acid Asp-Ala diketopiperazine (3-methyl-2,5-diketopiperazine-6-acetic acid, 5) had formed without any cross contamination.



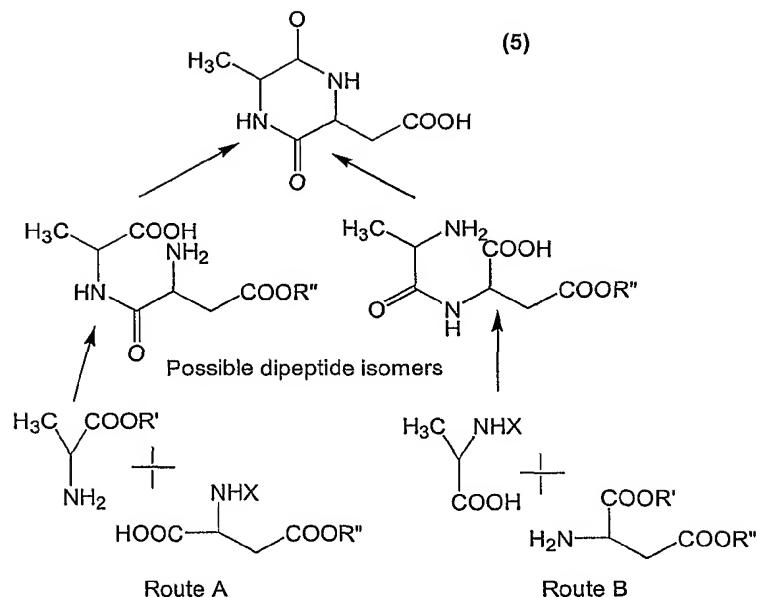
Example 2: Preparation Of Asp-Ala Diketopiperazine Amide (6)



5 To a solution of 3-methyl-2,5- diketopiperazine-6-acetic acid (0.151 g, 0.81 mmol, 1 equivalent, preparation described in Example 1, 5) in DMF (2.5 mL) was added carbonyl diimidazole (0.26 g, 1.60 mmol, 2 equivalents, Aldrich Chemical Co., Milwaukee, WI). After stirring at room temperature for 1 hour, solid ammonium acetate (0.63 g, 8.17 mmol, 10 equivalents, Aldrich) was added. Stirring at room temperature was continued overnight, 10 at which time the reaction was partitioned between water (20 mL) and ethyl acetate (10 mL). The aqueous layer was washed with a second aliquot of ethyl acetate (10 mL), and it was then evaporated to dryness under reduced pressure (61°C). Traces of DMF were removed by further co-evaporations with water and then toluene to give a white solid (362 mg). This was taken up into a minimum volume of methanol in DCM (20:80 v/v) and flushed through a 15 silica plug (10 g, chromatography grade silica; Aldrich) using methanol in DCM (20:80 v/v). The solvent eluted was fractionated, and the appropriate fractions were pooled and evaporated under reduced pressure (40°C) to give a white solid. The product was then recrystallized from methanol to give the desired product (0.116 g, 76% yield, 6).

20 Example 3: Alternate Syntheses of Asp-Ala Diketopiperazine

Essentially there are two ways that Asp-Ala diketopiperazine could be prepared, and the two processes are shown schematically below.



In this diagram, R', R'', and X are protecting groups.

In Route A, the free  $\alpha$ -carboxyl of the alanine is capable of undergoing an intramolecular cyclization with the free amino functional group of the protected aspartic acid. Route B describes the other alternative, whereby the free amino moiety of alanine cyclizes with the free carboxyl of the C-protected aspartic acid.

Solid phase synthesis (resin), as well as solution phase peptide chemistry, will give the dipeptide isomers, and both of these will yield the Asp-Ala diketopiperazine (5) upon cyclization. The method described in Example 1 is a Route A synthesis. Asp-Ala diketopiperazine has also been synthesized by Route B, and the spectrometric and chromatographic behaviors of the Asp-Ala diketopiperazine synthesized by the two Routes are identical.

The method described in Example 1 (Route A) is preferred for several reasons. The Fmoc-protected, resin-bound alanine is easily synthesized and commercially available on a large scale. Thus, this procedure could easily be scaled up. Also, the overall production cost of Route A is very much lower than that of Route B.

#### Example 4: Preparation of Glu-Ala Diketopiperazine

Wang resin having Ala-Fmoc attached thereto (3 g, 2.52 mmol, 1 equivalent, NovaBiochem) was transferred to a clean round-bottom, 100 mL flask, and a solution of

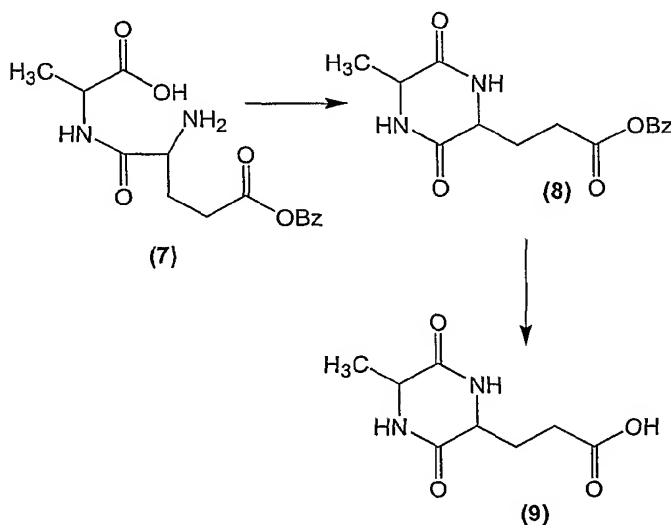
piperidine (12 mL) in DMF (18 mL) was added to the resin in the flask. The solution was swirled for 1 hour, and the resin was isolated in a sintered glass funnel. The resin was washed with DMF (3 x 30 mL) followed by DCM (3 x 30 mL) and allowed to dry under vacuum for 5 minutes. Preferred protecting groups are 9-fluorenylmethyloxycarbonyl (Fmoc) and t-butoxycarbonyl (Boc).

The partially dried resin was transferred into a clean round-bottom 100 mL flask and DMF (10 mL) was added. Then Boc-Glu(OBz)OH (3.40 g, 10.08 mmol, 4 equivalents, Sigma, St. Louis, MO) was added, followed by diisopropylamine (2.83 mL, 2.04 g, 20.19 mmol, 8 equivalents, Aldrich) and TBTU (3.24 g, 10.09 mmol, 4 equivalents, Acros). The slurry was allowed to react under anaerobic conditions for 10 hours. At the end of this time, the resin showed a negative ninhydrin test, indicating the completion of the coupling reaction. The resin was vacuum filtered and washed with DMF (3 x 30 mL) followed by DCM (3 x 30 mL). The resin was allowed to air dry at room temperature under vacuum for 10 minutes before transferring it into a clean round-bottom 100 mL flask.

TFA (16.5 mL) was added to the dried resin and, upon its addition, the resin turned a red color. After swirling the resin for a further 30 minutes, TFA was removed by filtration, and the resin was washed with DCM (4 x 20 mL). The organic components were pooled, and the combined organic materials were evaporated to a pale yellow paste under vacuum. Traces of TFA were removed by the addition of toluene and evaporation. The process was repeated until all TFA had been removed. This procedure resulted in a product as a pale yellow residue whose NMR and mass spectrophotometric data were consistent with the expected dipeptide benzyl ester whose structure (7) is shown below.

The dipeptide 7 was dissolved in butan-2-ol (40 mL) and diluted with toluene (60 mL). This solution was allowed to reflux for 18 hours. At the end of this period, the solution was allowed to cool to room temperature. It was then concentrated on a rotary evaporator while maintaining the temperature at 50°C. Upon concentration, a white solid was formed, and the precipitate was removed by filtration. The precipitate was washed with toluene (10 mL) and dried. The residue (0.683 g) gave a negative ninhydrin test. It was then crystallized from hot methanol. The spectroscopic and analytical results for the crystallized product confirmed its structure to be the desired compound – Glu-Ala-diketopiperazine-benzyl ester shown below (8).

This compound (500 mg) was dissolved in methanol (400 mL), and Pd/C (10%, 0.5 g) was added carefully. The flask was purged with hydrogen and kept at a positive hydrogen pressure. The solution was kept in this atmosphere for at least 7 hours. The catalyst was removed with a filtering aid (celite) and washed with methanol. The methanol washings were combined and the solvent was removed (yield 200 mg). Mass spectrometer and NMR analysis showed that the free acid Glu-Ala diketopiperazine (3-methyl-2,5-diketopiperazine-7-acetic acid; **9**) had formed without any cross contamination.



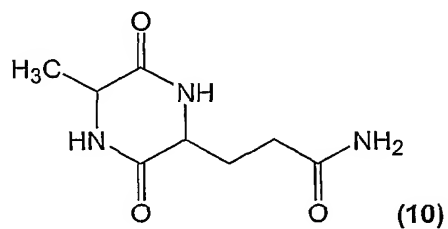
10

#### Example 5: Preparation of Glu-Ala Diketopiperazine Amide

To a solution of 3-methyl-2,5-diketopiperazine-7-acetic acid (0.153 g, 0.75 mmol, 1 equivalent, preparation described in Example 4, **9**) in DMF (2.5 ml) was added carbonyl diimidazole (0.24 g, 1.48 mmol, 2 equivalents, Aldrich). After stirring at room temperature for 1 hour, solid ammonium acetate (0.58 g, 7.52 mmol, 10 equivalents, Aldrich) was added. Stirring at room temperature was continued overnight, at which time the reaction was partitioned between water (20 ml) and ethyl acetate (10 ml). The aqueous layer was washed with a second aliquot of ethyl acetate (10 ml), and it was then evaporated to dryness under reduced pressure (56°C). Traces of DMF were removed by further co-evaporations with water and then toluene to give a white solid (317 mg). This was taken up into a minimum volume of methanol in DCM (20:80 v/v) and flushed through a silica plug using methanol

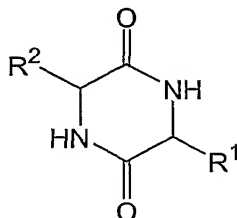
in DCM (20:80 v/v). The solvent eluted was fractionated, and the appropriate fractions were pooled and evaporated under reduced pressure (40°C) to give a white solid. The product was then recrystallized from methanol to give the desired product **10** (0.086 g, 57%).

5



WE CLAIM:

1. A method of synthesizing a diketopiperazine of the formula:



5

wherein:

$R^1$  is  $-\text{CH}_2\text{COR}^3$ , or  $-\text{CH}_2\text{CH}_2\text{COR}^3$ ;

$R^2$  is the side chain of an amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine;

$R^3$  is  $-\text{OH}$ ,  $-\text{NH}_2$ ,  $-\text{OR}^4$ ,  $-\text{NHR}^4$ , or  $-\text{NR}^4\text{R}^4$ ; and

each  $R^4$  is independently an alkyl, aryl, alkylaryl, or arylalkyl;

the method comprising:

(a) providing a first amino acid selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine, the first amino acid optionally being protected with one or more protecting groups so as to prevent unwanted side reactions;

(b) reacting the first amino acid with a second amino acid derivative having one of the following formulas:

$\text{NH}_2\text{CH}(\text{CH}_2\text{COOR}^5)\text{COOH}$  or

$\text{NH}_2\text{CH}(\text{CH}_2\text{CH}_2\text{COOR}^5)\text{COOH}$ ,

wherein:

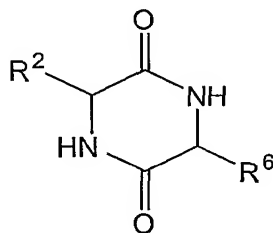
$R^5$  is a lower alkyl or alkylaryl, and

the amino group or  $\alpha$ -carboxyl of the second amino acid derivative is optionally protected with a protecting group so as to prevent unwanted side reactions,



the reaction taking place under conditions effective to form a dipeptide;

(c) cyclizing the dipeptide to form a diketopiperazine having the formula;



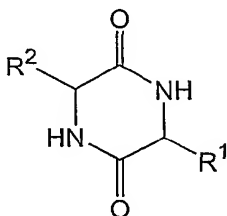
5 wherein:

$R^6$  is  $-\text{CH}_2\text{COOR}^5$ , or  $-\text{CH}_2\text{CH}_2\text{COOR}^5$ ; and

(d) removing the  $R^5$  group from the diketopiperazine to give a compound of formula 2 wherein  $R^3$  is  $-\text{OH}$ .

2. The method of Claim 1 wherein the first amino acid is alanine.
- 10 3. The method of Claim 1 wherein  $R^5$  is benzyl.
4. The method of Claim 2 wherein  $R^5$  is benzyl.
5. The method of Claim 1 wherein the dipeptide is cyclized by heating it under neutral conditions.
6. The method of Claim 5 wherein dipeptide is refluxed in a neutral solvent.
- 15 7. The method of Claim 6 wherein the neutral solvent is a combination of butan-2-ol and toluene.
8. The method of Claim 1 wherein the  $R^5$  group is removed by hydrogenation of the diketopiperazine formed in step (c).
9. The method of Claim 8 wherein the diketopiperazine of step (c) is
- 20 hydrogenated using a palladium on carbon catalyst.
10. The method of Claim 1 further comprising converting the product of step (d) to a compound of formula 2 wherein  $R^3$  is other than  $-\text{OH}$ .
11. A method of synthesizing a diketopiperazine of the formula:

25



wherein:

$R^1$  is  $-\text{CH}_2\text{COR}^3$ , or  $-\text{CH}_2\text{CH}_2\text{COR}^3$ ;

$R^2$  is the side chain of an amino acid selected from the group consisting of glycine,  
5 alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine;

$R^3$  is  $-\text{OH}$ ,  $-\text{NH}_2$ ,  $-\text{OR}^4$ ,  $-\text{NHR}^4$ , or  $-\text{NR}^4\text{R}^4$ ; and

each  $R^4$  is independently an alkyl, aryl, alkylaryl, or arylalkyl;

10 the method comprising:

(a) attaching a first amino acid to a solid support, the first amino acid being selected from the group consisting of glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartic acid, asparagine, glutamic acid, glutamine, lysine, hydroxylysine, histidine, arginine, phenylalanine, tyrosine, tryptophan, thyroxine, cysteine, methionine, norvaline and ornithine,  
15 the first amino acid optionally being protected with one or more protecting groups so as to prevent unwanted side reactions;

(b) reacting the first amino acid attached to the solid support with a second amino acid derivative having one of the following two formulas:

$\text{NH}_2\text{CH}(\text{CH}_2\text{COOR}^5)\text{COOH}$  or  
20  $\text{NH}_2\text{CH}(\text{CH}_2\text{CH}_2\text{COOR}^5)\text{COOH}$ ,

wherein:

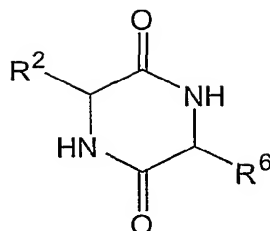
$R^5$  is a lower alkyl or alkylaryl, and

the amino group or  $\alpha$ -carboxyl of the second amino acid derivative optionally being protected with a protecting group so as to prevent unwanted side reactions,

25 the reaction taking place under conditions effective to form a dipeptide attached to the solid support;

(c) removing the dipeptide from the solid support;

(d) cyclizing the dipeptide to form a diketopiperazine having the formula:



wherein:

$R^6$  is  $-\text{CH}_2\text{COOR}^5$ , or  $-\text{CH}_2\text{CH}_2\text{COOR}^5$ ; and

5 (e) removing the  $R^5$  group to give a compound of formula 2 wherein  $R^3$  is  $-\text{OH}$ .

12. The method of Claim 11 wherein the first amino acid is alanine.

13. The method of Claim 11 wherein  $R^5$  is benzyl.

14. The method of Claim 12 wherein  $R^5$  is benzyl.

15. The method of Claim 11 wherein the dipeptide is cyclized by heating it under  
10 neutral conditions.

16. The method of Claim 15 wherein dipeptide is refluxed in a neutral solvent.

17. The method of Claim 16 wherein the neutral solvent is a combination of  
butan-2-ol and toluene.

18. The method of Claim 1 wherein the  $R^5$  group is removed by hydrogenation  
15 of the diketopiperazine formed in step (d).

19. The method of Claim 18 wherein the diketopiperazine of step (d) is  
hydrogenated using a palladium on carbon catalyst.

20. The method of Claim 11 further comprising converting the product of step (e)  
to a compound of formula 2 wherein  $R^3$  is other than  $-\text{OH}$ .

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/24281

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07D 241/08

US CL : 544/408

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 544/408

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
CASONLINE, EAST

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4,992,552 A (HUBBS et al.) 12 February 1991 (12.02.1991), See col. 1-2, formula II, Method B on col. 14-15, and Example 7.	1
---		-----
Y	See formula II on col. 2 and note the definition of R groups and Z groups include various amino acid side chains	2--10
X	US 5,932,579 A (CAMPBELL et al.) 03 August 1999 (03.08.1999), see entire document especially Table III, last entry.	11-20
---		-----
Y	See formula on col. 4 and R groups definition includes process for compounds of claims 1-20.	1-10
Y	EP 0 493 812 AI (AJINOMOTO CO., LTD.) 08 July 1990 (08.07.1990) See examples 1-7, especially examples 5 through 7.	1-20
Y	WO 91/14378 A1 (THE NUTRASWEET COMPANY) 03 October 1991 (03.10.1991). See entire document especially pages 9-10 and example 3.	1-20

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:		"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

25 September 2001 (25.09.2001)

Date of mailing of the international search report

20 NOV 2001

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks

Box PCT

Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

Venkataraman Balasubramanian

Telephone No. (703)308-1235